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RESEARCH ARTICLE

Toxicity Evaluation of Selenium Nanoparticles in Early Life Stages of Zebrafish

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ABSTRACT

The rapid advancement of nanotechnology necessitates innovative approaches to evaluate the safety of nanoscale materials. Zebrafish (Danio rerio) has emerged as a promising model for toxicity assessments due to their physiological and genetic similarities to vertebrates, rapid development, and transparent embryos. Selenium, a vital micronutrient with antioxidant and anticancer properties, is limited by its narrow therapeutic range, where supra-nutritional doses can be toxic. In various animal models, selenium nanoparticles (SeNPs) have been proposed as a less toxic alternative to traditional selenium forms, such as selenite. This study investigates the comparative toxicity of SeNPs, synthesized via L-ascorbic acid reduction, and sodium selenite on early life stages of zebrafish. Zebrafish embryos were exposed to varying concentrations of SeNPs and sodium selenite in aqueous solutions, with sodium dodecyl sulfate (SDS) serving as a negative control. Over 72 hours, key developmental parameters, including heartbeat, mortality, hatching, and malformation rates, were monitored at 24-hour intervals. Statistical analysis indicated that SeNPs exerted greater toxic effects on zebrafish embryos compared to selenite, as evidenced by higher rates of mortality and malformations. Notably, at low concentrations, both SeNPs and selenite enhanced hatching rates, suggesting a potential stimulatory effect. These findings underscore the need for careful consideration of SeNP toxicity in biomedical applications and highlight the utility of zebrafish as a model for nanotoxicology studies.

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INTRODUCTION

The growing application of nanomaterials and particles has led to public concern regarding their potential impact on the environment and human health. Their new properties appear in the nanoscale of numerous materials and might cause desirable and undesirable consequences during their interaction with bio-systems. As a result, evaluation and estimation of such outcomes should be considered before a nanomaterial enters the market. Contrary to bulk materials, whose concentration determines their role in assessing toxic effects, in the case of nanoparticles, in addition to concentration, multiple parameters such as size, shape, crystallinity, etc, regulate their behavior in biological systems and consequently modality of biological responses (1, 2).

Selenium, an essential trace element, has garnered significant interest for its potential role in the prevention and treatment of certain cancers due to its antioxidant properties and influence on cellular processes (3). While the antioxidant properties of selenium, an essential trace element, are well-established, ongoing debates persist regarding its efficacy in the treatment and prevention of various cancers, with conflicting evidence highlighting the need for further research(4) . Furthermore, research frequently indicates that supra-nutritional or higher doses of selenium, administered as selenite ions, selenomethionine, or other common supplemental forms, are often required to achieve

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therapeutic effects in cancer treatment (5). However, its narrow therapeutic window, cumulative effects from prolonged exposure, and potential toxicity at supra-nutritional doses raise significant concerns regarding its safe use in high-dose regimens (6). To address concerns about the toxicity of conventional selenium compounds, researchers have explored selenium nanoparticles (SeNPs) as a promising alternative for cancer therapy. Although elemental selenium is largely inert in biological systems, SeNPs exhibit notable bioactivity, potentially offering reduced toxicity and enhanced therapeutic efficacy (7). To evaluate the comparative toxicity of SeNPs and selenite ions as selenium-containing supplements, we utilized zebrafish (ZF) as an in vivo model for toxicological studies. Furthermore, the use of different selenium forms as dietary supplements in livestock, poultry, and aquaculture industries has been extensively discussed. Research indicates that supra-nutritional selenium levels can positively impact key industry metrics, such as Feed Conversion Ratio (FCR), highlighting its potential benefits in animal production systems (8).

Zebrafish (Danio rerio) have emerged as a highly valuable model organism in various biological research domains, particularly in genetics and toxicology, due to their numerous advantageous traits. Key characteristics, including the transparency of embryos and larvae, external fertilization, high fecundity, and cost-effective maintenance and breeding, facilitate detailed studies of developmental processes. Moreover, the physiological similarities in response to xenobiotics between zebrafish and humans, coupled with the heightened sensitivity of zebrafish embryos to chemical exposures compared to other life stages, enable clear detection of chemical effects. These attributes establish zebrafish as an effective model for toxicological assessments and developmental toxicology studies(9, 10) . Endorsed by the National Institutes of Health (NIH) and the National Institute of Environmental Health Sciences (NIEHS), zebrafish are recognized as a robust alternative model for advancing research on human health and environmental toxicology, capitalizing on their vertebrate-like genetic and physiological traits (11). Also, a study on zebrafish can help to increase fish farming efficiency. Due to greater reactivity, it is generally accepted that smaller particles might show more serious toxicity (12-14); however, regarding SeNPs some controversial evidence reveals its lower toxicity in animal models (15-17). Confirmation of these assertions in other species upholds the possibility of the prescription of higher doses of selenium for therapeutic purposes without concerns regarding its unpleasant side effects. Due to growing SeNPs applications, such as optoelectronic, sensors, medical imaging, and solar cells (18, 19), leading to more undesirable interactions between SeNPs and the human body as well as its probable release into the environment, it spurred us to evaluate its toxicity and other effects in terms of hatching rate, heart rate, the incidence of mortality, and visible abnormalities in early life stages of zebrafish.

METHOD

Synthesis of selenium nanoparticles

SeNPs were synthesized at ambient temperature in a volumetric flask. A 40 mL aqueous solution containing 2.5 mg/mL selenium dioxide (SeO_2, Merck, Germany) and 0.25 mg/mL sodium dodecyl sulfate (SDS, Merck, Germany) was combined with 40 mL of a 10 mg/mL aqueous L-ascorbic acid solution (Kimia Mavad, Iran). The reaction mixture was subsequently diluted to a final volume of 100 mL with deionized water (Milli-Q, Merck, Germany) to yield the SeNPs suspension.

Purification of nanoparticles

To purify sodium dodecyl sulfate (SDS)-stabilized nanoparticles, the suspension was subjected to centrifugation (Sigma 3- 30KS, Germany) at 10,000 rpm for 10 minutes, followed by removal of the supernatant. The resulting pellet was resuspended in deionized water, and this centrifugation-resuspension cycle was repeated three times to eliminate water-soluble impurities, including residual SDS, unreacted selenium dioxide (SeO_2), L-ascorbic acid, and dehydroascorbic acid, a reaction byproduct. For enhanced dispersion, the suspension was ultrasonicated (Bandelin HD 2070, Germany) for 20 minutes. The purified nanoparticles were then lyophilized (Christ Alpha 1-2 LDplus, Germany) to obtain a dry powder for subsequent use.

Characterization of nanoparticles

The physicochemical properties of synthesized SeNPs were comprehensively characterized to evaluate their size, size distribution, surface charge, elemental composition, morphology, and crystalline structure. The hydrodynamic diameter, size distribution, and zeta potential of SeNPs in a 1

mg/mL aqueous suspension were determined using dynamic light scattering (DLS) with a Microtrac NANO-flex instrument (Microtrac, USA). The morphology, size, and elemental composition of the SeNPs were analyzed using field-emission scanning electron microscopy (FESEM, TE-Scan MIRA3, Czech Republic) coupled with energy-dispersive X-ray spectroscopy (EDX). For FESEM analysis, a single drop of the SeNPs suspension was air-dried on an aluminum substrate to facilitate imaging. The crystalline structure of the SeNPs powder was characterized using X-ray diffraction (XRD) with a Bruker D8-Advance diffractometer (Bruker, Germany).

Fish farming and embryo production

To generate zebrafish embryos for experimental studies, a controlled breeding aquarium was established in our laboratory. Forty adult wild-type zebrafish were procured from a certified local supplier and acclimated in a 60-liter aquarium. The aquarium was filled with dechlorinated tap water, processed through a household filtration system to remove impurities, and maintained at a temperature of 28.5 ± 0.5°C and a pH range of 7.5–8.0. Water quality was further optimized by the addition of 60 mg/L sea salt (Instant Ocean) to mimic natural ionic conditions. Aeration was provided to ensure adequate oxygenation. The fish were fed a balanced commercial fish diet twice daily and maintained on a 14-hour light/10-hour dark photoperiod to regulate their circadian rhythm.

For breeding, male and female zebrafish were transferred to a specialized spawning tank the evening prior to embryo collection, at a sex ratio of 2:1 (males:females). Spawning was induced the following morning upon activation of the light cycle, facilitating natural mating and fertilization. Viable embryos were promptly collected and gently rinsed in a washing medium composed of sterilized tap water supplemented with 60 mg/L sea salt (Instant Ocean). To ensure sterility, a dilute solution of sodium hypochlorite was briefly applied as a disinfectant, followed by thorough rinsing with the washing medium to eliminate residual chemical traces. The prepared embryos were then staged and maintained under controlled conditions for subsequent experimental use.

SeNPs exposure assessment

Zebrafish embryos at the blastula stage (3–4 hours post-fertilization) were used to evaluate the toxicity of SeNPs and sodium selenite (Na_2SeO_3, Merck, Germany) as a source of selenite ions. Four concentrations (1.25, 2.5, 5, and 10 µg/mL) of SeNPs suspensions and sodium selenite solutions were prepared by serial dilution in a culture medium formulated to mimic aquarium water. Embryos were distributed into 12-well plates, with seven embryos per well. One plate was designated for SeNPs solutions and another for selenite solutions, with three replicate wells per concentration. Additionally, three wells per plate were assigned to 0.01% SDS as a capping agent for SeNPs, and three wells contained only culture medium as negative controls. Each well received 2 mL of the respective solution, and the plates were incubated at 28.5°C for 72 hours. Developmental endpoints were assessed at 24, 48, and 72 hours post-fertilization (hpf) using a stereomicroscope (Nikon SMZ800N, Japan). At each time point, heart rate, hatching rate, mortality rate, and visible morphological abnormalities were recorded. Dead embryos and larvae were removed during each observation to maintain experimental integrity.

Data analysis

All experiments were conducted in triplicate to ensure reproducibility. Data were expressed as mean ± standard deviation (SD) and analyzed using IBM SPSS Statistics (Version 24, SPSS Inc., USA). To evaluate significant differences between the experimental groups and the control, one-way analysis of variance (ANOVA) was performed, followed by the Least Significant Difference (LSD) post-hoc test. Additionally, an independent t-test was used to compare the effects of equivalent concentrations of SeNPs and selenite ions. For both statistical tests, a p-value of less than 0.05 was considered indicative of statistical significance.

RESULTS

Upon initiation of the reaction, the solution exhibited an immediate color transition to orange, which progressively deepened to a dark red hue over time. This coloration remained stable for approximately three months, indicating the formation and sustained stability of the synthesized product.

Characterization of SeNPs

Scanning electron microscopy (SEM) analysis revealed that SeNPs exhibit a spherical morphology (Figure 1A). DLS measurements indicated an

average particle diameter of 31.50 nm, with approximately 85% of the particles falling within the size range of 22.5 to 35.0 nm. The particle size distribution was characterized by a polydispersity index (PDI) of 0.085, indicating high uniformity (Figure 1B). Additionally, the zeta potential (ZP) of the SeNPs was measured at -53.17 mV, suggesting excellent colloidal stability. XRD analysis of the SeNPs powder confirmed the crystalline structure (Figure 1C). EDS revealed prominent peaks corresponding to selenium (SeLα) and elements associated with SDS used in the synthesis process (Figure 1D).

Effects of SeNPs and selenite on embryos and larvae

Monitoring heart rate (HR, beats per minute, bpm) in zebrafish embryos and larvae exposed to SeNPs, sodium selenite, SDS, and untreated controls revealed consistent temporal patterns across most groups. Specifically, HR exhibited a marked increase at 48 hpf, followed by a decline at 72 hpf, with values at 72 hpf remaining slightly elevated compared to those at 24 hpf. However, significant differences were observed between the control and SeNPs-treated groups. At SeNPs concentrations of 5 µg/mL and 10 µg/mL, a notable reduction in HR was detected compared to the control, particularly at 48 hpf (112 ± 10 bpm for 5 µg/mL, 108 ± 13 bpm for 10 µg/mL, versus 156 ± 3 bpm for control) and 72 hpf (95 ± 13 bpm for 5 µg/mL, 122 ± 10 bpm for 10 µg/mL, versus 138 ± 5 bpm for control). In contrast, no statistically significant differences in HR were observed among the selenite-treated, SDS-treated, and control groups (see Figure 2). These findings suggest that SeNPs exert a more pronounced effect on cardiac function in zebrafish embryos and larvae compared to selenite, potentially indicating greater toxicity at the tested concentrations.

At 24 hpf, embryos across all experimental groups, including the control, SeNPs-treated, selenite-treated, and SDS-treated groups, exhibited normal developmental progression, with an overall mortality rate below 4.5%. However, by 72 hpf, significant differences in mortality were observed between the SeNPs-treated groups and the control. Specifically, mortality rates in SeNPs-treated embryos were 26.99 ± 7.32% at 1.25 µg/mL, 28.57 ± 6.30% at 2.5 µg/mL, 65.08 ± 11.69% at 5 µg/mL, and 74.60 ± 10.85% at 10 µg/mL, compared to 12.70 ± 3.72% in the control group. In contrast, selenite-treated embryos at equivalent concentrations showed no statistically significant differences in mortality compared to the control at either 48 or 72 hpf (Figure 3). These findings indicate that SeNPs exert a greater toxic effect on zebrafish embryos than selenite at higher concentrations over the 72-hour exposure period.

Evidence demonstrates that low doses of selenium, in the forms of SeNPs and selenite, exert

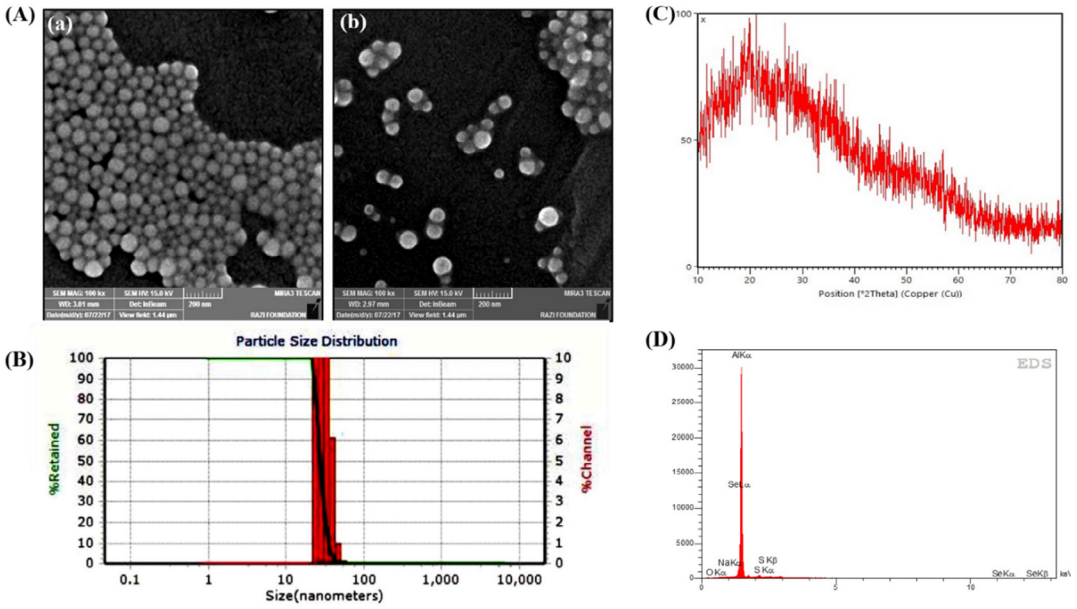

Fig. 1. (A). SEM images of SeNPs in two magnifications (a, b), (B). Particle size distribution of SeNPs determined by DLS, (C). XRD pattern of SeNPs powder and (D). EDX analysis of SeNPs.

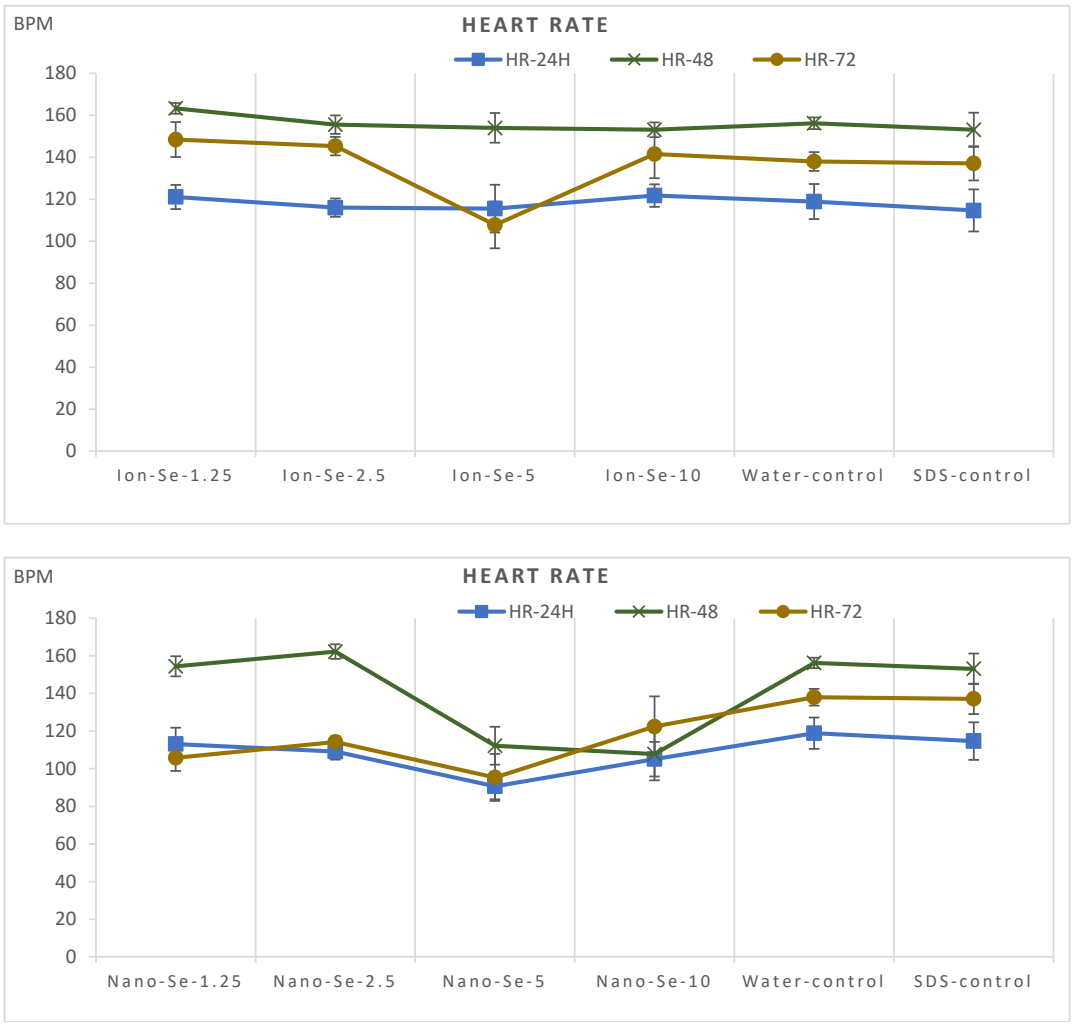

Fig. 2. The rate of heart beat (BPM) of Zebrafish embryos and larvae at each checked time-point (24, 48 & 72hpf) and indicated concentrations (1.25, 2.5, 5 & 10 µg/ml) of Se-ion (up) and Nano-Selenium (down) and comparison with controls. Results are presented as mean±SD.

a stimulatory effect on the hatching time and rate of zebrafish embryos. Specifically, at 48 hpf, both SeNPs and selenite at a concentration of 2.5 µg/mL significantly enhanced the hatching rate compared to the control group (30.16% ± 10.50 for SeNPs-treated, 22.22% ± 5.89 for selenite-treated, versus 3.80% ± 2.10 for control). However, by 72 hpf, SeNPs exhibited concentration-dependent toxic effects, leading to a significant reduction in hatching rates at 5 µg/mL (79.36% ± 3.70) and 10 µg/mL (61.37% ± 9.59) compared to the control (96.30% ± 2.45). In contrast, selenite across all tested concentrations showed no significant adverse impact on hatching rates at 72 hpf. The SDS-treated group displayed hatching rates (93.38% ± 2.63) comparable to the control, indicating minimal interference with hatching processes (Figure 4). These findings highlight the dual role of SeNPs, with beneficial effects at low concentrations but pronounced toxicity at higher doses, underscoring the need for careful dose optimization in potential applications.

During the experiment, several types of developmental abnormalities were observed and monitored, including tail deformities, spinal column curvature, pericardial edema, and significant indicators of growth retardation, such as the failure to deplete the yolk sac (Figure 5).

After 48 hpf, only the SeNPs-treated samples at a concentration of 10 µg/mL exhibited a significant increase in abnormal phenotypes (4.76% ± 3.37) compared to the control (0.00%). At 72 hpf, in addition to the SeNPs-treated samples at 10 µg/

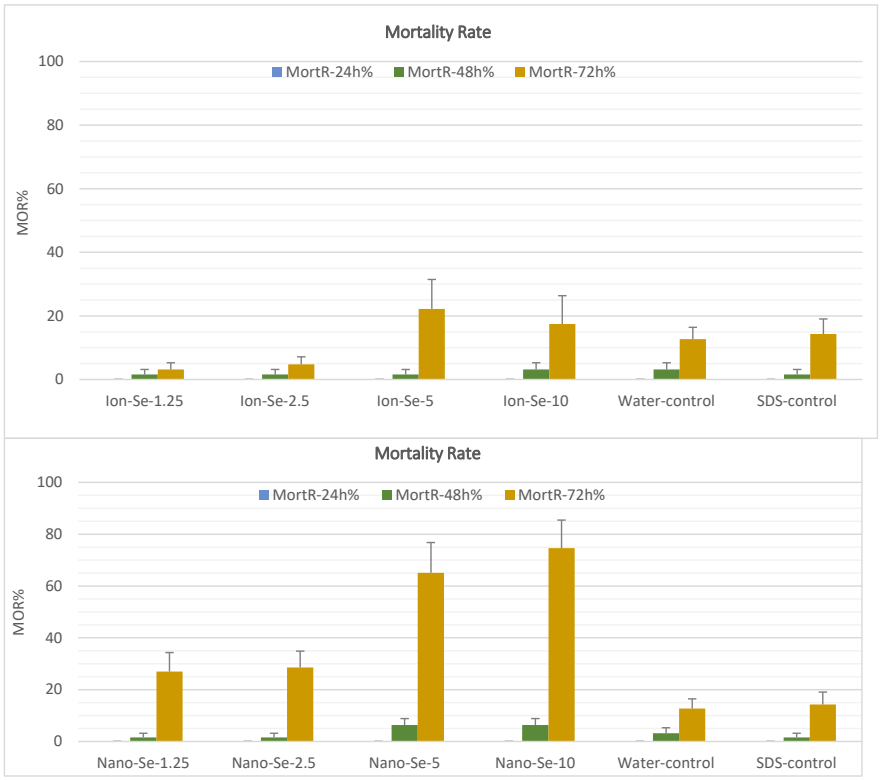

Fig. 3. The mortality rate of Zebrafish embryos and larvae at different checked time-point (24, 48 & 72 hpf) and indicated concentrations (1.25, 2.5, 5 & 10 µg/ml) of Se-ion(up) & SeNPs (down) and comparison with controls. Results are presented as mean±SD.

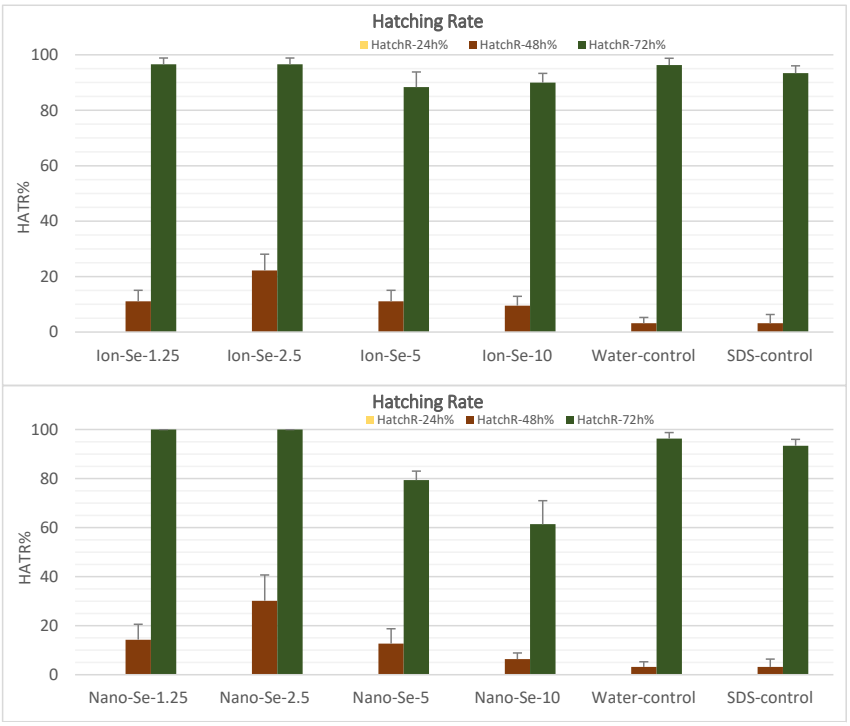

Fig. 4. The hatching rate of Zebrafish embryos at different checked time-point (24, 48 & 72 hpf) and indicated concentrations (1.25, 2.5, 5 & 10 µg/ml) of Se-ion(up) and SeNPs (down) and comparison with controls. Results are presented as mean±SD.

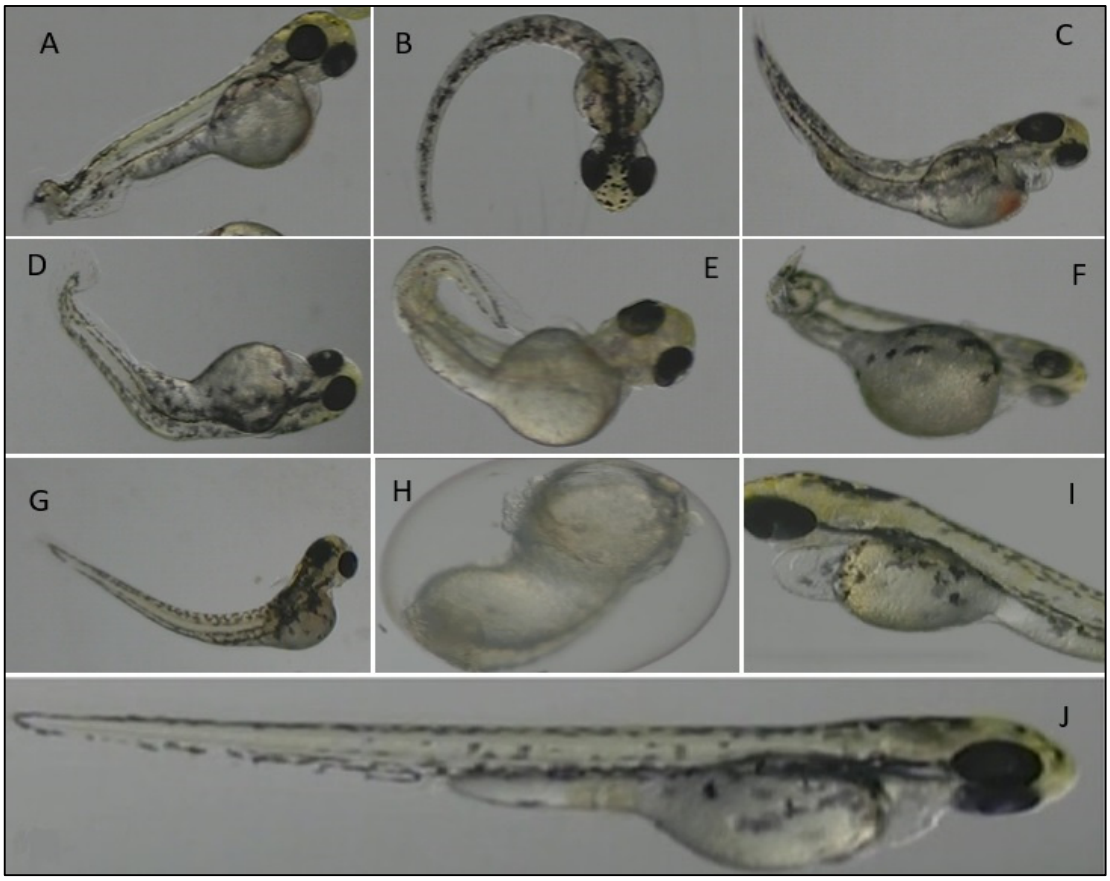

Fig. 5. Different malformations were observed in ZF larvea after 72h. A: Tail malformation related to 10µg/ml selenite ion, B,C: realated to 5µg/ml concentration of SeNPs. D,E,F and G: Show spinal column curveture, growth retardation and non depletion of yolk related to 10µg/ml concentration of SeNPs. H: An alive embryo with severe growth retardation 72hpf. I: A larva with pericardial edema. J: A normal ZF larva 72hpf.

mL (7.94% ± 2.51), the selenite-treated samples at the same concentration also displayed significant malformations (7.94% ± 3.46) compared to the control (0.00%) (Figure 6).

DISCUSSION

Numerous methods have been employed for the synthesis of SeNPs. Among these, the reduction of selenium ions, particularly selenite, using a reducing agent, followed by stabilization with appropriate materials, represents one of the most straightforward approaches to produce SeNPs with diverse sizes and morphologies (20). Stabilizing agents and reaction conditions, including agent concentration and temperature, significantly influence the size, morphology, and stability of NPs. Ascorbic acid (vitamin C), acting as a mild reducing agent, undergoes two-stage oxidation to form hydrated dehydroascorbic acid (DHAA·H_2O). During this process, it reduces selenious acid (H_2SeO_3), the soluble form of SeO_2, to elemental selenium (Se^0). The resulting Se^0 precipitates as nanoparticles, typically organized in eight-atom ring structures, causing a rapid color change in the solution due to nanoparticle formation (21). SDS was a stabilizer to prevent nanoparticle (NP) aggregation. The PDI and particle size distribution graph (Figure 1) indicated that the SeNPs suspension was monodisperse. Furthermore, the PDI, average particle size, and SEM data strongly correlated. The EDS confirmed that the NPs consisted solely of selenium and SDS. The amorphous structure of the SeNPs was inferred from the red color of the suspension and substantiated by the XRD pattern of the powdered sample. To assess the direct effects of SeNPs on zebrafish embryos, nanoparticle size was controlled due to the chorionic pore size of approximately

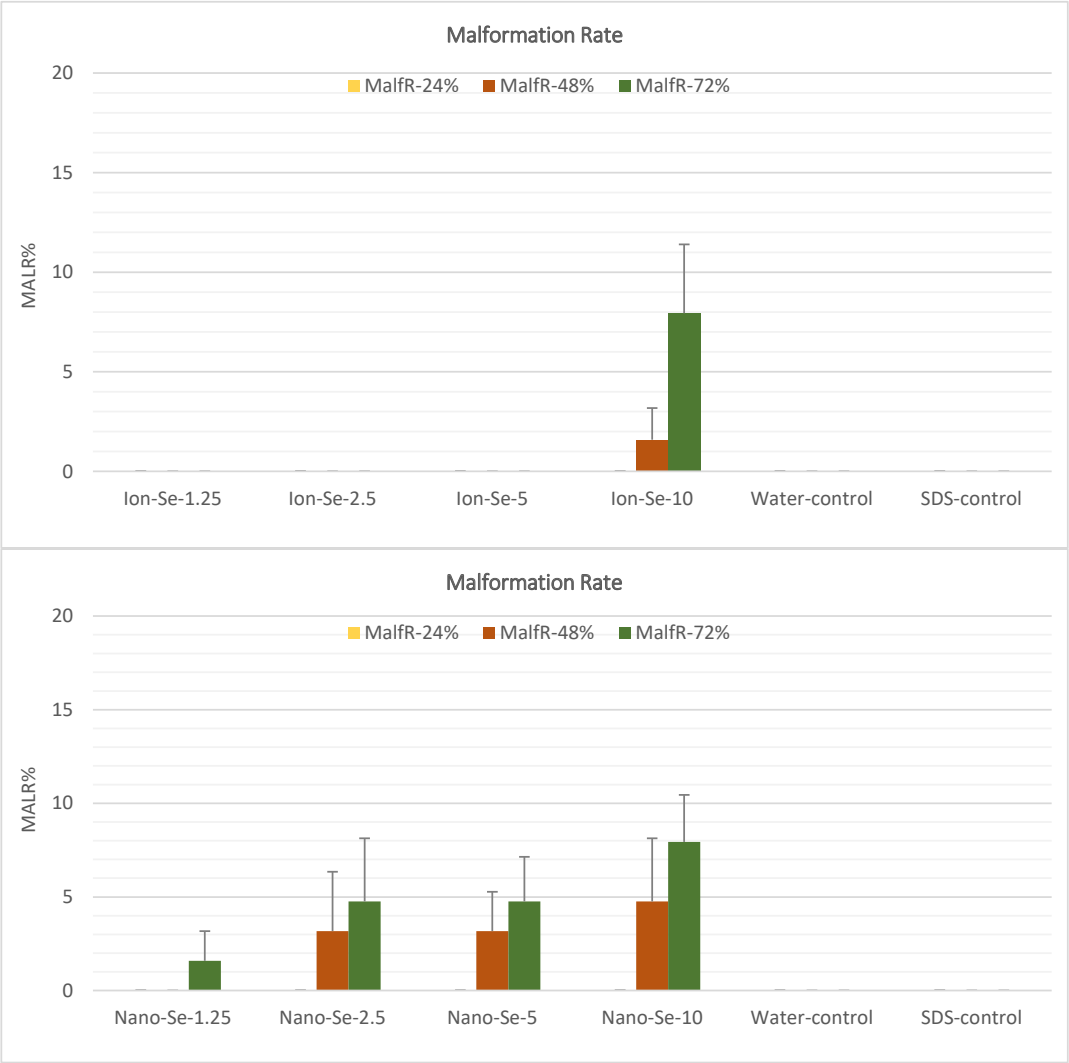

Fig. 6. The malformation rate of Zebrafish embryos and larvae at different checked time-point (24, 48 & 72 hpf) and indicated concentrations (1.25, 2.5, 5 & 10 µg/ml) of SeNPs (down) and Se-ion(up) and comparison with controls. Results are presented as mean±SD.

0.5–0.7 µm, which restricts the entry of particles of this size or larger (22).

Some studies indicate that NPs, even those with diameters as small as 60 nm, are unable to penetrate this barrier (23). The synthesis of smaller SeNPs was a key objective to facilitate their passive diffusion across cellular membranes. Regarding toxicity, comparative studies between selenite and other selenium forms are frequently conducted. Research involving mice, rats, and birds has demonstrated that SeNPs exhibit significantly lower toxicity compared to other selenium-containing compounds, such as selenite and selenate ions. These findings highlight the potential of SeNPs as a safer therapeutic and adjuvant option for cancer treatment (24). Based on the findings of this study, SeNPs demonstrated greater toxicity compared to selenite across all evaluated parameters, particularly at concentrations of 5 and 10 µg/mL.

The selection of zebrafish as a model organism in this study was driven by its close genetic homology to humans and its established utility in developmental and aquatic toxicity assessments, which are closely linked to human health concerns (25). While the toxicological effects of SeNPs have been explored in other Actinopterygii species, such as Japanese medaka, this study represents a novel investigation into the comparative toxicity of SeNPs and selenite ions in the early life stages of zebrafish, providing new insights into their differential impacts.

Selenium toxicity primarily arises from its

interference with disulfide bond formation, critical for protein folding, due to its chemical similarity to sulfur. At high concentrations, selenium forms selenotrisulfide (S-Se-S) or selenenylsulfide (S-Se) bonds, disrupting protein function (26, 27). Additionally, inorganic selenium reacts with thiols like glutathione, generating selenotrisulfides and superoxide radicals that induce oxidative stress (28, 29). For SeNPs, toxicity is heavily influenced by particle size, which affects bioavailability and cellular interactions (29). Studies on crucian carp intestinal cells and medaka fish have shown that smaller SeNPs (e.g., 36 nm) exhibit higher toxicity due to hyper-accumulation and slow clearance compared to selenite (30, 31). A similar implication has been reported in broiler chickens (32). However, this study found similar malformation phenotypes in both SeNP- and selenite-treated zebrafish, suggesting shared metabolic pathways despite differing toxicological profiles.

Selenium's cardioprotective effects, mediated through mechanisms like the prostacyclin/thromboxane ratio, are well-documented, as is its ability to mitigate chemotherapy-induced toxicity (33, 34). However, at toxic doses, selenium can induce cardiotoxicity in fish, manifesting as pericarditis, myocarditis, and bradycardia (35, 36). In this study, SeNPs at 5 and 10 µg/mL caused significant heart rate reductions and mild pericardial edema in zebrafish embryos and larvae at 72 hpf, unlike selenite, which showed negligible effects at equivalent doses. These findings align with prior research indicating that larger SeNPs (500–600 nm) at higher concentrations (20–25 µg/mL) induce similar cardiotoxic effects, including blood congestion and edema. The pronounced cardiotoxicity of SeNPs appears to stem from their nanostructure rather than their chemical composition, highlighting the need for further mechanistic studies.

Low-dose selenium, whether as selenite or SeNPs (e.g., 2.5 µg/mL), enhanced zebrafish hatching rates, likely due to its role in promoting cell proliferation, suggesting shared metabolic pathways (37-40). This effect, while not inherently beneficial, holds potential for improving productivity in aquaculture and ornamental fish industries by reducing hatching time. However, conflicting studies report that selenium supplementation primarily enhances meat quality and antioxidant capacity rather than growth (41-43), though supra-nutritional doses may reduce morbidity and mortality in commercial settings (44). Developmental malformations, including spinal curvature, tail deformities, non-depleted yolk sacs, and growth retardation, were prominent indicators of selenium toxicity in this study, consistent with prior findings in fish (35, 36, 45). In contrast to Kalishwaralal et al., who observed significant toxicity at 20–25 µg/mL with larger SeNPs (500 nm) after 96 hpf, this study detected effects at lower concentrations, likely due to the smaller size (31.50 nm) and higher bioavailability of our SeNPs (28, 46). These results underscore the critical role of nanoparticle size in toxicity and the necessity for tailored safety assessments in biomedical and environmental applications.

CONCLUSION

This study elucidates the comparative toxicity of SeNPs and sodium selenite in early life stages of zebrafish, revealing that SeNPs exhibit significantly higher toxicity at concentrations of 5 and 10 µg/mL. Key indicators, including increased mortality, reduced heart rates, impaired hatching, and elevated developmental malformations, underscore the potential risks of SeNPs, likely due to their nanoscale properties and bioaccumulation. In contrast, selenite showed minimal toxicity, suggesting a safer profile. However, at lower concentrations (e.g., 2.5 µg/mL), both compounds enhanced hatching rates, indicating a hormetic effect with potential applications in aquaculture, provided safe dosages are established.

These findings highlight the critical need for careful evaluation of SeNPs in biomedical and environmental contexts, given their heightened toxicity compared to traditional selenium forms. The zebrafish model proved highly effective for these assessments, leveraging its transparency and physiological similarities to vertebrates. Future research should focus on mitigating SeNPs toxicity, defining safe exposure thresholds, and exploring molecular mechanisms to ensure their safe use in therapeutic and industrial applications while minimizing ecological risks.

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DECLARATIONS

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The authors have no conflicts of interest to declare that are relevant to the content of this article.

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